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For:	EMBRYONIC STEM CELLS	**Dated:**	February 25, 2002

Assistant Commissioner for Patents
Washington, DC 20231

CLAIM OF PRIORITY

Sir:

Applicants in the above-identified application hereby claim the right of priority in connection with Title 35 U.S.C. §119 and in support thereof, herewith submits certified copies of Australian Patent Application No. PP7009 filed on November 9, 1998 and Australian Patent Application No. PQ2852 filed on September 15, 1999.

Respectfully submitted,

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CERTIFICATE OF MAILING UNDER 37 C.F.R. §1.8(a)

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on February 25, 2002.

Dated: February 25, 2002

Anna Baerga

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AUSTRALIA

Patent Office
Canberra

I, LEANNE MYNOTT, MANAGER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PP 7009 for a patent by MONASH UNIVERSITY, NATIONAL UNIVERSITY OF SINGAPORE and NATIONAL UNIVERSITY HOSPITAL filed on 09 November 1998.

WITNESS my hand this
Fourth day of December 2001

LEANNE MYNOTT
MANAGER EXAMINATION SUPPORT
AND SALES

P/00/009
Regulation 3.2

AUSTRALIA
Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: EMBRYONIC STEM CELLS

Applicant: MONASH UNIVERSITY and
NATIONAL UNIVERSITY OF SINGAPORE

The invention is described in the following statement:

EMBRYONIC STEM CELLS

The present invention relates to undifferentiated embryonic stem cells, methods of cultivation and propagation, production of differentiated cells and uses thereof.

The promotion of stem cells capable of being maintained in an undifferentiated state *in vitro* allows for the study of the cellular and molecular biology of early human development, functional genomics, generation of differentiated cells from the stem cells for use in transplantation or drug screening and drug discovery *in vitro*.

In general, stem cells are undifferentiated cells which can give rise to a succession of mature functional cells. For example, a haematopoietic stem cell may give rise to any of the different types of terminally differentiated blood cells. Embryonic stem (ES) cells are derived from the embryo and are pluripotent, thus possessing the capability of developing into any organ to tissue type or, at least potentially, into a complete embryo.

Mouse ES cells have successfully been maintained in an undifferentiated state using fibroblast feeder layers in cultivation in the presence of leukaemia inhibitory factor (LIF). If LIF is removed, mouse ES cells will differentiate.

Mouse ES cells are indeed different to human ES cells. This is evident from the requirements necessary to maintain the cells in an undifferentiated state. Whilst fibroblast feeder layers or LIF are sufficient to prevent differentiation of mouse ES cells and allows continuous passage, this is insufficient for human ES cells. High concentrations of cloned LIF fail to prevent differentiation of primate ES cells in the absence of fibroblast feeder layer. Pluripotent human embryonal carcinoma cells and cells isolated from the human blastocyst similarly cannot be serially cultivated in the presence of LIF alone. Cultivation of undifferentiated human ES cells has previously failed to produce more than a couple of passages before differentiation becomes evident.

It is an object of the invention to overcome or at least alleviate some of the problems of the prior art.

SUMMARY OF THE INVENTION

In one aspect of the present invention, there is provided a purified preparation of human embryonic stem cells capable of proliferation *in vitro* for an extended period.

Preferably the cells have the potential to differentiate when subjected to differentiating conditions.

More preferably, they are capable of maintaining an undifferentiated state when cultured on a fibroblast feeder layer.

In another aspect of the present invention there is provided an undifferentiated human embryonic stem cell wherein the cell is immunoreactive with markers for human pluripotent stem cells including SSEA-4, GCTM-2 antigen, TRA 1-60 and CD30. Preferably, the cells express the transcription factor Oct-4 as demonstrated by RT-PCR. More preferably, the cells maintain a diploid karyotype during prolonged cultivation *in vitro*.

In a further aspect of the present invention there is provided a method of preparing undifferentiated human embryonic stem cells, said method including:

obtaining *in vitro* fertilised human embryo;

removing inner cell mass (ICM) cells from the embryo;

culturing ICM cells on a fibroblast feeder layer to obtain stem cells; and

removing stem cells from the feeder layer.

In a preferred aspect of the invention the method further includes the following steps before removal of inner cell mass cells, said steps including:

treating the embryo to dislodge the trophectoderm of the embryo or a portion thereof;

washing the embryo with an appropriate blastocysts culture medium; for example G2 or S2 (Scandinavian-2 medium) to dislodge the trophectoderm or a portion thereof; and

obtaining inner cell mass cells of the embryo.

Preferably, the treatment of the embryo includes treating with an antibody or antiserum reactive with epitopes on the surface of the trophectoderm. More preferably, the treatment with antibody or antiserum is combined with treatment with complement. Most preferably, the combined

antibody and complement are either anti-placental alkaline phosphatase antibody combined with Baby Rabbit complement; or antihuman serum antibody combined with Guinea Pig complement. The antibody and complement may be used together or separately to treat the embryo to dislodge the trophectoderm or a portion thereof.

In a further aspect of the invention, the method further includes:

replacing the stem cells from the fibroblast feeder layer onto another fibroblast feeder layer; and

culturing the stem cells for a period sufficient to obtain morphologically undifferentiated stem cells.

In an even further aspect of the invention the method further includes propagating the undifferentiated stem cells. The methods of propagation may initially involve dispersing stem cells from clumps representing colonies of cells. The dispersion is preferably by chemical or mechanical means. More preferably, the cells are treated chemically and washed in a Ca^{2+}/Mg^{2+} free PBS or they are mechanically severed from the colonies or a combination of the two methods.

In another aspect there is provided an undifferentiated cell line produced by the method of the present invention.

In another aspect of the invention there is provided a method of induction of differentiation of stem cells.

In a further aspect of the invention, there is provided a method of producing large quantities of differentiated cells.

FIGURES

Figure 1 shows a colony of undifferentiated human ES cell line HES-1.

Figure 2 shows a colony from the same cell line which has undergone differentiation.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect of the present invention there is provided a purified preparation of human undifferentiated embryonic stem cells capable of proliferation *in vitro* for an extended period of time.

The extended period may include several passages of cells. The cells substantially maintain in an undifferentiated state.

Preferably the cells have the potential to differentiate when subjected to differentiating conditions.

More preferably, they are capable of maintaining an undifferentiated state when cultured on a fibroblast feeder layer.

The promotion of stem cells capable of being maintained in an undifferentiated state *in vitro* allows for the study of the cellular and molecular biology of early human development, functional genomics, generation of differentiated cells from the stem cells for use in transplantation or drug screening and drug discovery *in vitro*. Until now, successful maintenance of the human stem cells in an undifferentiated state has not been achieved successfully.

Once the cells are maintained in the undifferentiated state, they may be differentiated to mature functional cells. The embryonic stem cells are derived from the embryo and are pluripotent and have the capability of developing into any organ or tissue type. Preferably the tissue type is selected from the group including blood cells, neuron cells or muscle cells.

The cells undergo differentiation *in vitro* to yield somatic cells as well as extrembryonic cells, such differentiation being characterised by novel gene expression characteristic of specific lineages as demonstrated by immunocytochemical or RNA analysis.

Differentiating cultures of the stem cells secrete HCG and AFP into culture medium, as determined by enzyme-linked immunosorbent assay carried out on culture supernatants.

In another aspect of the present invention there is provided an undifferentiated human embryonic stem cell wherein the cell is immunoreactive with markers for human pluripotent stem cells including SSEA-4, GCTM-2 antigen, TRA 1-60, CD30. Preferably, the cells express the transcription factor Oct-4 as demonstrated by RT-PCR. More preferably the cells maintain a diploid karyotype during prolonged cultivation *in vitro*.

Preferably, the stem cell will constitute a purified preparation of an undifferentiated stem cell line. More preferably, the stem cell line is a permanent cell line, distinguished by the characteristics identified above. They preferably have normal karyotype along with the characteristics identified above. This combination of defining properties will identify the cell lines of the invention regardless of the method used for their isolation.

Methods of identifying these characteristics may be by any method known to the skilled addressee. Methods such as (but not limited to) indirect immunoflourescence or immunocytochemical staining may be carried out on colonies of ES cells which are fixed in ethanol and are dried using colonies of ES cells which are fixed by conventional fixation protocols then stained using antibodies against stem cell specific antibodies and visualised using secondary antibodies conjugated to fluorescent dyes or enzymes which can produce insoluble colored products. Alternatively, RNA may be isolated from the stem cells and PR-PCR or Northern blot analysis carried out to determine expression of stem cell specific genes.

In a preferred embodiment the undifferentiated cells form tumours when injected in the testis of immoundeprived SCID mice; these tumours include differentiated cells representative of all three germ layers. The germ layers are preferably endoderm, mesoderm and ectoderm. Preferably, once the tumours are established, they may be disassociated and specific differentiated cell types may be identified or selected by any methods available to the skilled addressee. For instance, lineage specific markers may be used through the use of fluorescent activated cell sorting (FACS) or other sorting method or by direct micro dissection of tissues of interest. These differentiated cells may be used in any manner. They may be cultivated *in vitro* to produce large numbers of differentiated cells which could be used for transplantation or for use in drug screening for example.

In a further aspect of the present invention there is provided a method of preparing undifferentiated human embryonic stem cells, said method including:

obtaining an *in vitro* fertilised human embryo;

removing inner cells mass (ICM) cells from the embryo;

culturing ICM cells on a fibroblast feeder layer to obtain stem cells; and

removing stem cells from the feeder layer.

Embryonic stem cells (ES) are derived from the embryo. These cells are undifferentiated and have the capability of differentiation to a variety of cell types. The "embryo" is defined as the first developmental stage after fertilisation. It develops from repeated division of cells and includes the stages of a blastocyst stage which comprises an outer trophectoderm and an inner cell mass (ICM).

The embryo required in the present method is an *in vitro* fertilised embryo.

The embryo may be fertilised by any *in vitro* methods available. For instance, the embryo may be fertilised by using conventional insemination, or intracytoplasmic sperm injection. It is preferred that any embryo culture method is employed but it is most preferred that a method producing high quality (good morphological grade) of blastocysts is employed. The high quality of the embryo can be assessed by morphological criteria. Most preferably the inner cell mass is well developed. These criteria can be assessed by the skilled addressee.

Following insemination, embryos may be cultured to the blastocyst stage. This stage may be assessed to determine suitable embryos for deriving ICM cells. The embryos may be cultured in any medium that maintains their survival and enhances blastocyst development.

Preferably, the embryos are cultured in droplets under pre-equilibrated in a suitable medium such as G1, S1 or sterile mineral oil in IVF-50 medium (Scandinavian IVF). Preferably the incubation is for two days. On approximately the third day, an appropriate medium may be used such as a mixture of 1:1 of IVF-50 and Scandinavian-2 medium (Scandinavian IVF) may be used. From at least the fourth day, a suitable medium such as G2 or S2 Scandinavian-2 medium may be used solely to grow the embryos to blastocyst stage (blastocysts).

In a preferred embodiment, the blastocyst is subjected to enzymatic digestion to remove the zona pellucida or a portion thereof. Preferably the blastocyst is subjected to the digestion at an expanded blastocyst stage. Generally this is at approximately six days after insemination.

Any protein enzyme may be used to digest the zona pellucida or portion thereof from the blastocyst. Examples include pronase, acid Tyrodes solution, and mechanical methods such as laser dissection.

Preferably, Pronase is used. The pronase may be dissolved in PBS and G2 or S2 medium Scandinavian-2 medium. Preferably the PBS and Scandinavian-2 medium is diluted 1:1. For digestion of zone pellucida from the blastocyst, approximately 10 units of Pronase may be used for a period sufficient to remove the zona pellucida. Preferably approximately 1 to 2 mins, more preferably 1-1.5 mins is used.

The embryo (expanded blastocyst) may be washed in G2 or S2 medium (Scandinavian-2) medium, and further incubated to dissolve the zona pellucida. Preferably, further digestion steps may be used to completely dissolve the zona. Removal of the zona pellucida thereby exposes the ICM (and trophectoderm (TE)).

In a preferred aspect of the invention the method further includes the following steps before removal of inner cell mass cell, said steps including:

treating the embryo to dislodge the trophectoderm of the embryo or a portion thereof;

washing the embryo with a G2 or S2 (Scandinavian-2) medium to dislodge the trophectoderm or a portion thereof; and

obtaining inner cell mass cells of the embryo.

Having had removed the zona pellucida, the ICM and trophectoderm become accessible. Preferably the trophectoderm is separated from the ICM. Any method may be employed to separate the trophectoderm from the ICM. Preferably the embryo (or blastocyst devoid of zona pellucida) is subjected to immuno-surgery. Preferably it is treated with an antibody or antiserum reactive with epitopes on the surface of the trophectoderm. More preferably, the treatment of the embryo, (preferably an embryo at the blastocyst stage devoid

of zona pellucida) is combined with treatment with complement. The antibody and/or antiserum and complement treatment may be used separately or together. Preferred combinations of antibody and/or antiserum and complement include anti-placental alkaline phosphatase antibody and Baby Rabbit complement (Serotec) or anti-human serum antibody (Sigma) combined with Guinea Pig complement (Gibco).

Preferably the antibodies and complement are diluted in G2 or S2 (Scandinavian-2) medium. The antibodies and complement, excluding anti-placental alkaline phosphate (anti-AP) are diluted 1:5 whereas anti-AP antibody is diluted 1:20 with Scandinavian-2 medium.

Preferably the embryo or blastocyst (preferably having the zona pellucida removed) is subjected to the antibody before it is subjected to the complement. Preferably, the embryo or blastocyst is cultured in the antibody for a period of approximately 30 mins.

Following the antibody exposure, it is preferred that the embryo is washed. Preferably it is washed in G2 or S2 (Scandinavian-2) medium. The embryo or blastocyst preferably is then subjected to complement, preferably for a period of approximately 30 mins.

G2 or S2 (Scandinavian-2) medium is preferably used to wash the embryo or blastocyst to dislodge the trophectoderm or a portion thereof. Dislodgment may be by mechanical means. Preferably the dislodgment is by pepetting the blastocyst through a small bore pipette.

The ICM cells may then be exposed and ready for removal and culturing. Culturing of the ICM cells is conducted on a fibroblast feeder layer. In the absence of a fibroblast feeder layer, the cells will differentiate. Leukaemia inhibitory factor (LIF) has been shown to replace the feeder layer in some cases and maintain the cells in an undifferentiated state. However, this seems to only work for mouse cells. For human cells, high concentration of LIF were unable to maintain the cells in an undifferentiated state in the absence of a fibroblast feeder layer.

Mouse or human fibroblasts are preferably used. They may be used separately or in combination. Human fibroblasts provide support for stem cells,

but they create a non-even and sometimes non-stable feeder layer. Human fibroblasts are mostly preferred. However, they may combine effectively with mouse fibroblasts to obtain an optimal stem cell growth and inhibition of differentiation.

The cell density of the fibroblast layer affects its stability and performance. A combination of mouse and human fibroblasts is preferred. A density of approximately 25,000 human and 70,000 mouse cells per cm^2 is most preferred. The fibroblasts may be plated up to 48 hours before culturing the stem cells. Mouse fibroblasts alone are used at 100,000/cm^2. The feeder layers are preferably established 4-24 hours prior to addition of ES cells.

Preferably the mouse or human fibroblast cells are low passage number cells. The quality of the fibroblast cells affects their ability to support the stem cells. Embryonic fibroblasts are preferred. For mouse cells, they may be obtained from 12 day old foetuses. However, any source is suitable. Human fibroblasts may be derived from embryonic of foetal tissue from termination of pregnancy and may be cultivated using standard protocols of cell culture.

It is preferred that the cells are treated to arrest their growth. Several methods are available. It is preferred that they are irradiated or are treated with chemicals such as mitomycin C which arrests their growth. Most preferably, the fibroblast feeder cells are treated with mitomycin C.

The fibroblast feeder layer maybe generally plated on a geletin treated dish. Preferably, the tissue culture dish is treated with 0.1% gelatin.

The fibroblast feeder layer may also contain modified fibroblasts. For instance, fibroblasts expressing recombinant membrane bound factors essential for stem cell renewal may be used. Such factors maybe CD30 ligand or Jagged 1 or human multipotent stem cell factor.

Inner cell mass cells may be cultured on the fibroblast feeder layer and maintained in an ES medium. A suitable medium is DMEM (GIBCO, without sodium pyruvate, with glucose 4500mg/L) supplemented with 20% FBS (Hyclone, Utah), (betamercaptoethanol - 0.1mM (GIBCO), non essential amino acids - NEAA 1% (GIBCO), glutamine 2mM. (GIBCO), and penicillin 50µ/ml, streptomycin 50µg/ml (GIBCO). In the early stages of ES cell cultivation, the

medium maybe supplemented with human recombinant leukemia inhibitory factor hLIF preferably at 2000μ/ml. However, LIF generally is not necessary. Any medium may be used that can support the ES cells.

The ES medium may be further supplemented with soluble growth factors which promote stem cell growth or survival or inhibit stem cell differentiation. Examples of such factors include human multipotent stem cell factor, or GM-CSF, or embryonic stem cell renewal factor.

The isolated ICM may be cultured for at least six days. At this stage, clumps of cells or colonies of cells develop. These colonies are clumps of principally undifferentiated stem cells. They may exist on top of differentiated cells. Isolation of the undifferentiated cells may be achieved by chemical or mechanical means of both. They may be removed mechanically by a micropipette or they maybe dispensed in a Ca^{2+}/Mg^{2+} free PBS medium or both.

In a further aspect of the invention, the method further includes:

replating the stem cells from the fibroblast feeder layer onto another fibroblast feeder layer; and

culturing the stem cells for a period sufficient to obtain morphologically undifferentiated stem cells.

A further replating of the undifferentiated stem cells is preferred. The isolated clumps of cells from the first fibroblast feeder layer may be replated on fresh human/mouse fibroblast feeder layer as described above.

Preferably, the cells are cultured for a period of 10-14 days. After this period, colonies of undifferentiated stem cells may be observed. The stem cells may be morphologically identified preferably by the high nuclear/cytoplasmic ratios, prominent nucleoli and compact colony formation. The cell borders are often distinct and the colonies are often flatter than mouse ES cells. The colonies resemble those formed by pluripotent human embryonal carcinoma cell lines such as GCT 27 X-1.

Other means of identifying the stem cells may be by cell markers. Preferably the stem cells may be identified by being immunoreactive with markers for human pluripotent stem cells including SSEA-4, GCTM-2 antigen,

TRA 1-60, CD30. Preferably the cells express the transcription factor oct-4 as demonstrated by RT-PCR. The cells may also maintain a diploid karyotype.

In an even further aspect of the invention, the method further includes propagating the undifferentiated stem cells. The methods of propagation may initially involve dispersing stem cells from clumps representing colonies of cells. The dispersion is preferably by chemical or mechanical means. More preferably, the cells are washed in a Ca/Mg free PBS or they are mechanically severed from the colonies or a combination of the two methods. In both methods cells which appeared nondifferentiated may be propagated as clumps of about 50 cells every 5-7 days.

In the first method, Ca^{2+}/Mg^{2+} free PBS medium may be used to reduce cell-cell attachments. Following about 15-20 minutes, cells gradually start to dissociate from the monolayer and from each other and desired size clumps can be isolated. When cell dissociation is partial, mechanical dissociation using the sharp edge of the pipette may assist with cutting and the isolation of the clumps.

An alternative chemical method may include the use of an enzyme. The enzyme may be used alone or in combination with a mechanical method. Preferably, the enzyme is dispase.

An alternative approach includes the combined use of mechanical cutting of the colonies followed by isolation of the subcolonies by dispase. Cutting of the colonies may be performed in PBS containing Ca^{2+} and Mg^{2+}. The sharp edge of a micropipette may be used to cut the colonies to clumps of about 75-100 cells. The pipette may be used to scrape and remove differentiated areas of the colonies. The PBS is preferably changed to regular equilibrated human stem cell medium containing dispase (Gibco) 10 mg/ml and incubated for approximately 5-10 minutes at 37°C in a humidified atmosphere containing 5% CO_2. As soon as the clumps detached they may be picked up by a wide bore micro-pipette, washed in PBS containing Ca^{2+} and Mg^{2+} and transferred to a fresh feeder layer.

The feeder layer may be as described above.

The stem cells may be further modified at any stage of isolation. They may be genetically modified through introduction of vectors expressing a selectable marker under the control of a stem cell specific promoter such as Oct-4 or CD30. These cells may be selected against differentiated cells. The presence of such cells may further induce stem cell differentiation.

The stem cells may be genetically modified at any stage with markers so that the markers are carried through to any stage of cultivation. The markers may be used to purify the undifferentiated stem cell population at any stage of cultivation.

Progress of the stem cells and their maintenance in an undifferentiated stage may be monitored in a quantitative fashion by the measurement of stem cell specific secreted products into the culture medium or in fixed preparations of the cells using ELISA or related techniques. Such stem cell specific products might include the soluble form of the CD230 antigen or the GCTM-2 antigen.

In another aspect of the invention there is provided a method of induction of differentiation of stem cells.

Undifferentiated ES cells in the right conditions will differentiate into the embryonic germ layers (endoderm, mesoderm and ectoderm). However, this differentiation process can be controlled. Preferably the timing of the cell type can be controlled.

The undifferentiated cell lines of the present invention may be cultured indefinitely until a differentiating signal is given.

Preferably, a differentiating signal inducing differentiation includes cultivating to high density. Once achieving confluence (ie. continuously covering the culture surface) the cells may spontaneously differentiate.

Differentiation may also be induced by cultivating to a high density in monolayer or on semi-permeable membranes so as to create structures mimicing the postimplantation phase of human development, or any modification of this approach. Cultivation in the presence of cell types representative of those known to modulate growth and differentiation in the vertebrate embryo (eg. endoderm cells or cells derived from normal embyronic or neoplastic tissue) may also induce differentiation.

Chemical differentiation may also be used to induce differentiation. Propagation in the presence of soluble or membrane bound factors known to modulate differentiation of vertebrate embryonic cells, such as bone morphogenetic protein-2 may be used.

Selective cultivation may be a means of controlling the induction of differentiation in the stem cells.

Genetic modification of the stem cells or further modification of those genetically modified stem cells described above may be employed to control the induction of differentiation. Genetic modification of the stem cells so as to introduce a construct containing a selectable marker under the control of a promoter expressed only in specific cell lineages, followed by treatment of the cells as described above and the subsequent selection for cells in which that promoter is active may be used.

In another aspect there is provided an undifferentiated cell line produced by the method of the present invention.

A specific cell line is HES-1 isolated by the procedures described above and having the properties described above.

In another aspect of the invention there is provided a cell composition including a human undifferentiated cell line preferably produced by the method of the present invention, and a carrier.

The carrier may be any physiologically acceptable carrier that maintains the cells. It may be PBS or ES medium.

The undifferentiated cells may be used as a source for isolation or identification of novel gene products, or for the generation of antibodies against novel epitopes. The cell lines may be used for the development of means to diagnose prevent or treat congenital diseases.

The present invention will now be more fully described with reference to the following examples. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

Finally it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

DATED: 9 November, 1998

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David B Fitzpatrick

FIGURE 1

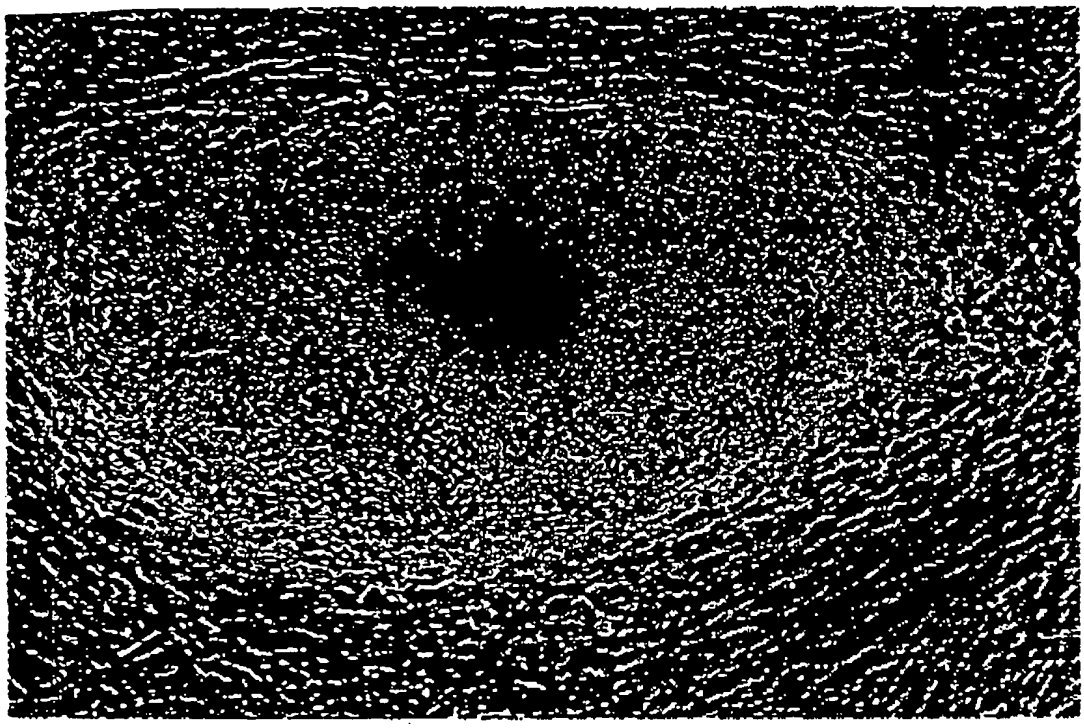

FIGURE 2